SLOVENSKI STANDARD
SIST EN 13623:2010

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Kemična razkužila in antiseptiki - Kvantitativni suspenzijski preskus za vrednotenje baktericidnega delovanja kemičnih razkužil za razkuževanje vode na bakterijo Legionella - Preskusna metoda in zahteve (faza 2, stopnja 1)

Chemical disinfectants and antiseptics - Quantitative suspension test for the evaluation of bactericidal activity against Legionella of chemical disinfectants for aqueous systems - Test method and requirements (phase 2, step 1)

Chemische Desinfektionsmittel und Antiseptika - Quantitativer Suspensionsversuch zur Bestimmung der bakteriziden Wirkung gegen Legionella von chemischen Desinfektionsmitteln für wasserführende Systeme - Prüfverfahren und Anforderungen (Phase 2, Stufe 1)

Antiseptiques et désinfectants chimiques - Essai quantitatif de suspension pour l'évaluation de l'activité bactéricide contre des légionelles des désinfectants chimiques pour les systèmes aqueux - Méthode d'essai et prescriptions (phase 2, étape 1)

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Foreword

This document (EN 13623:2010) has been prepared by Technical Committee CEN/TC 216 “Chemical disinfectants and antiseptics”, the secretariat of which is held by AFNOR.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by March 2011, and conflicting national standards shall be withdrawn at the latest by March 2011.

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Introduction

This European Standard specifies a suspension test for establishing whether a chemical disinfectant has a bactericidal activity against *Legionella pneumophila* in the fields described in the scope. This standard is specifically prepared for water treatment products, but it may also be possible to use it for other products.

Proliferation of *Legionella* only occurs in waters under certain conditions, and predominantly poses a risk when aerosolised. Many systems containing water do not require treatment. A decision to add chemical disinfectants to any water should be based on a risk assessment.

If the product complies with the requirements of this standard, it can be considered bactericidal against *Legionella pneumophila*, but it should not necessarily be inferred that the product is acceptable for a specific site of application without consideration of other relevant factors such as the pH, water, chemistry, temperature and degree of biological fouling at that site of application. It does not take into account the protective effect conveyed by biofilm on the organisms.

The conditions are intended to cover general purposes and to allow reference between laboratories and product types. Each concentration of the chemical disinfectant found by this test corresponds to defined experimental conditions. However, for some applications the recommendations of use of a product may differ and therefore additional test conditions need to be used.

1 Scope

This European Standard specifies a test method and the minimum requirements for bactericidal activity of chemical disinfectant products intended to be used for treatment in aqueous systems against *Legionella pneumophila* that form a homogeneous, physically stable preparation when diluted with buffered ferrous hard water or hard water. Whenever *Legionella pneumophila* poses a risk to human health, this method is suitable for water used in cooling towers and water for general purposes, like spas, pools, showers and other uses. The method is not suitable for electro-chemical disinfection.

The European Standard applies to products used to treat water in order to kill *Legionella pneumophila.*

NOTE 1 The method described is intended to determine the activity of commercial formulations or active substances under the conditions in which they are used.

NOTE 2 This method corresponds to a phase 2 step 1 test .

NOTE 3 This method does not take into account the fact that *Legionella pneumophila* is often found in cells of amoebae and/or biofilms and that thereby a product's activity against the bacteria may be reduced.

EN 14885 specifies in detail the relationship of the various tests to one another and to "use recommendation".

2 Normative references

The following referenced documents are indispensable for the application of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 14885, *Chemical disinfectants and antiseptics — Application of European Standards for chemical disinfectants and antiseptics*

3 Terms and definitions

For the purposes of this document, the terms and definitions given in EN 14885 and the following apply.

3.1
cooling water
water used to remove heat from a process or environment

3.2
water for general purposes
water used in premises other than water used as cooling water

4 Requirements

The product shall demonstrate al least a four decimal log (lg) reduction, when diluted with buffered ferrous hard water (5.2.2.10) or hard water (5.2.2.7), and tested in accordance with Clause 5 under the obligatory test conditions (one selected test organism, at either 20 °C or 30 °C) within 60 min for rapid acting products or 15 h for slower acting products.

The bactericidal activity shall be evaluated using the following test organism: *Legionella pneumophila.*

Where indicated, additional specific bactericidal activity shall be determined applying other contact times and test organisms (in accordance with 5.2.1 and 5.5.1.1) in order to take into account intended specific use conditions.

NOTE For these additional conditions, the concentration defined as a result can be lower than the one obtained under the obligatory test conditions.

5 Test methods

5.1 Principle

5.1.1 A sample of the product diluted with hard water (5.2.2.7 or 5.2.2.10) is added to a test suspension of bacteria in a solution of an interfering substance. The mixture is maintained at either (20 ± 1) °C or (30 ± 1) °C for 60 min ± 10 s or (15 ± 1) h (obligatory test conditions). At the end of the chosen contact time, an aliquot is taken, and the bactericidal and/or the bacteriostatic activity in this portion is immediately neutralized or suppressed by a validated method. The method of choice is dilution-neutralization. If a suitable neutralizer cannot be found membrane filtration is used. The numbers of surviving bacteria in each sample are determined and the reduction is calculated.

5.1.2 The test is performed using *Legionella pneumophila* as test organism (obligatory test conditions).

5.1.3 Additional and optional contact times are specified. Additional test organisms may be used.

5.2 Materials and reagents

5.2.1 Test organism

The bactericidal activity shall be evaluated using the following strain as test organism [1]: *Legionella pneumophila*: serogroup 1, Philadelphia (NCTC 11192; ATCC 33152).

If required for specific applications, additional test organisms may be used, e.g. *Legionella pneumophila* serogroup 1 Benidorm (NCTC 12006, ATCC 43108).

The required incubation temperature for this test organism is (36 ± 1) °C or (37 ± 1) °C (5.3.2.3). The same temperature (either 36 °C or 37 °C) shall be used for all incubations performed during a test and its control and validation.

If additional test organisms are used, they shall be incubated under optimum growth conditions (temperature, time, atmosphere, media) noted in the test report. If the additional test organisms selected do not correspond to the specified strains, their suitability for supplying the required inocula shall be verified. If these additional test organisms are not classified at a reference centre, their identification characteristics shall be stated. In addition, they shall be held by the testing laboratory or national culture collection under a reference for five years.

5.2.2 Culture media and reagents

5.2.2.1 General

Unless specifically stated, all weights of chemical substances given in this standard refer to the anhydrous salts. Hydrated forms may be used as an alternative, but the weights required shall be adjusted to allow for consequent molecular weight differences.

The reagents shall be of analytical grade and/or appropriate for microbiological purposes. They shall be free from substances that are toxic or inhibitory to the test organisms.

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1) The NCTC and ATCC numbers are the collection numbers of strains supplied by the National Type Culture Collection (NCTC) and American Type Culture Collection (ATCC). This information is given for the convenience of users of this standard and does not constitute an endorsement by CEN of the product named.

NOTE 1 To improve reproducibility, it is recommended that commercially available dehydrated material is used for the preparation of culture media. The manufacturer's instructions relating to the preparation of these products should be rigorously followed.

NOTE 2 For each culture medium and reagent, a limitation for use should be fixed.

5.2.2.2 Water

The water shall be freshly glass-distilled water and not demineralized water. If distilled water of adequate quality is not available, water for injections (bibliographic reference [1]) can be used.

Sterilize in the autoclave (5.3.2.1, a)). Sterilization is not necessary if the water is used e.g. for preparation of culture media and subsequently sterilized.

NOTE See 5.2.2.10 for the procedure to prepare buffered ferrous hard water.

5.2.2.3 Buffered Charcoal Yeast Extract (BCYE) Agar

BCYE agar, consisting of

—	yeast extract (bacteriological grade)	10,0 g;
—	agar	12,0 g;
—	activated charcoal	2,0 g;
—	alpha-ketoglutarate, monopotassium salt	1,0 g;
—	ACES buffer (N-2-acetamido-2-aminoethanesulfonic acid)	10,0 g;
—	potassium hydroxide (KOH) (pellets)	2,8 g;
—	L-cysteine hydrochloride monohydrate	0,4 g;
—	iron(III) pyrophosphate [Fe4(P207)3]	0,25 g;
—	distilled water	to 1 000,0 ml.

Preparation

a) Cysteine and iron solutions

Prepare fresh solutions of L-cysteine hydrochloride and iron(III) pyrophosphate by adding 0,4 g and 0,25 g respectively to 10-ml-volumes of water (5.2.2.2). Sterilize each solution by membrane filtration (5.3.2.7). Store in clean sterile containers at (20 ± 3) °C for not more than three months.

b) ACES buffer

Add the ACES granules to 500 ml of water (5.2.2.2) and dissolve by standing in a water bath at 45 °C to 50 °C. To a separate 480 ml of water (5.2.2.2), add all the potassium hydroxide pellets and dissolve with gentle shaking. To prepare the ACES buffer, mix the two solutions.

NOTE 1 ACES buffer can cause denaturation of the yeast extract if the following sequence is not followed.

c) Final medium

Add sequentially to the 980 ml of ACES buffer, the charcoal, yeast extract and α-ketoglutarate. Prepare a 0,1 mol/l solution of potassium hydroxide (KOH) by dissolving 5,6 g in 1 l of water (5.2.2.2). Prepare a 0,1 mol/l solution of sulphuric acid (H_2SO_4) by carefully adding 5,3 ml of H_2SO_4 to 1 l of water (5.2.2.2). Use the solutions of 0,1 mol/l potassium hydroxide or 0,1 mol/l sulphuric acid as appropriate to adjust the pH to 6,9 ± 0,2. Add the agar, mix and autoclave (5.3.2.1, a)). After autoclaving, allow to cool to (47 ± 2) °C in a water bath (5.3.2.2).

Add the L-cysteine and the iron(III) pyrophosphate solutions aseptically, mixing well between additions.

Dispense in 20 ml volumes into Petri dishes of 90 mm to 100 mm diameter. The pH of the final medium is 6,9 ± 0,2 at 25 °C. Allow excess moisture on the plates to dry and store at (4 ± 2) °C in airtight containers in the dark for up to four weeks.

Prolonged heating during sterilisation or heating at too high a temperature shall be avoided, as it can affect the nutritional qualities of BCYE medium. Batch-to-batch variation of the ingredients of the medium (particularly α-ketoglurarate) can also affect its performance. Therefore it is essential to check the quality of each newly prepared batch of media for its ability to support the growth of the test organism within three days of incubation using the validation suspension N_V (5.4.1.5).

NOTE 2 The ability of the media to support the growth of Legionella should be assessed quantitatively using either known quantities of the obligatory Legionella strain or by direct comparison to previous batches. Commercially supplied media may be used without testing if it has been performance tested in a laboratory accredited to EN ISO/IEC 17025:2005 for that purpose.

5.2.2.4 BCYE Broth

Prepared by the same method as BCYE agar (5.2.2.3), but omitting the addition of the agar.

5.2.2.5 Neutralizer

The neutralizer shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.2. It shall be sterile.

NOTE Information on neutralizers that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.6 Rinsing liquid (for membrane filtration)

The rinsing liquid shall be validated for the product being tested in accordance with 5.5.1.2, 5.5.1.3 and 5.5.3. It shall be sterile, compatible with the filter membrane and capable of filtration through the filter membrane under the test conditions described in 5.5.3.

NOTE Information on rinsing liquids that have been found to be suitable for some categories of products is given in Annex B.

5.2.2.7 Hard water for general purposes (HWGP)

For the preparation of 1 l of hard water, the procedure is as follows:

a) prepare solution A: dissolve 19,84 g magnesium chloride ($MgCl_2$) and 46,24 g calcium chloride ($CaCl_2$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave (5.3.2.1, a)). Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month;

b) prepare solution B: dissolve 35,02 g sodium bicarbonate ($NaHCO_3$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;

c) place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be 7,0 ± 0,2, when measured at (20 ± 1) °C (5.3.2.4). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl).

The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

NOTE 1 When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

NOTE 2 This hard water represents typical conditions of non cooling water.

5.2.2.8 Interfering substance (yeast extract)

— yeast extract 0,5 g;

— water to 1 000,0 ml.

Sterilize in the autoclave (5.3.2.1, a)).

Final concentration of the yeast extract in the test is 0,000 5 %.

5.2.2.9 Page's Saline

Saline solution, consisting of:

— sodium chloride (NaCl) 0,120 g;

— magnesium sulphate ($MgSO_4 \times 7H_20$) 0,004 g;

— calcium chloride ($CaCl_2 \times 2H_20$) 0,004 g;

— disodium hydrogen phosphate (Na_2HPO_4) 0,142 g;

— potassium dihydrogenphosphate (KH_2PO_4) 0,136 g;

— water (5.2.2.2) to 1 000,0 ml.

Sterilize in the autoclave (5.3.2.1, a)).

NOTE To aid accurate preparation, it is recommended that a 10 l volume of Page's Saline is prepared and dispensed in smaller volumes as required for autoclaving. Alternatively the salt solutions may be made up individually in concentrated form for dilution when the product is required.

5.2.2.10 Buffered ferrous hard water for treatment of cooling water (BFHW)

For the preparation of 1 l of BFHW, the procedure is as follows:

a) prepare solution A: dissolve 19,84 g magnesium chloride ($MgCl_2$) and 46,24 g calcium chloride ($CaCl_2$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7) or in the autoclave (5.3.2.1, a)). Autoclaving – if used – may cause a loss of liquid. In this case make up to 1 000 ml with

water (5.2.2.2) under aseptic conditions. Store the solution in the refrigerator (5.3.2.8) for no longer than one month;

b) prepare solution B: dissolve 35,02 g sodium bicarbonate ($NaHCO_3$) in water (5.2.2.2) and dilute to 1 000 ml. Sterilize by membrane filtration (5.3.2.7). Store the solution in the refrigerator (5.3.2.8) for no longer than one week;

c) place 600 ml to 700 ml of water (5.2.2.2) in a 1 000 ml volumetric flask (5.3.2.12) and add 70 ml of 0,2 M boric acid (13,6 g of Boric acid made up to 1 000 ml with distilled water), 30 ml of 0,05 M borax (19,07 g of Borax made up to 1 000 ml with distilled water) and 6,0 ml (5.3.2.9) of solution A, then 8,0 ml of solution B. Finally add 1,0 ml ferric sulphate solution (3,0 x 10^{-3} mol/l). Mix and dilute to 1 000 ml with water (5.2.2.2). The pH of the hard water shall be 8,0 ± 0,2, when measured at (20 ± 1) °C (5.3.2.4). If necessary, adjust the pH by using a solution of approximately 40 g/l (about 1 mol/l) of sodium hydroxide (NaOH) or approximately 36,5 g/l (about 1 mol/l) of hydrochloric acid (HCl). Sterilize by membrane filtration (5.3.2.7).

The hard water shall be freshly prepared under aseptic conditions and used within 12 h.

NOTE 1 When preparing the product test solutions (5.4.2), the addition of the product to the hard water produces a different final water hardness in each test tube. In any case the final hardness is lower than 300 mg/l of calcium carbonate ($CaCO_3$) in the test tube.

NOTE 2 This buffered ferrous hard water represents typical conditions of cooling water.

5.3 Apparatus and glassware

5.3.1 General

Sterilize all glassware and parts of the apparatus that will come into contact with the culture media and reagents or the sample, except those that are supplied sterile, by one of the following methods:

a) By moist heat, in the autoclave (5.3.2.1, a));

b) By dry heat, in the hot air oven (5.3.2.1, b)).

5.3.2 Usual microbiological laboratory equipment[2)]

In particular, the following:

5.3.2.1 Apparatus for sterilization

a) for moist heat sterilization, an autoclave capable of being maintained at (121^{+3}_{0}) °C for a minimum holding time of 15 min;

b) for dry heat sterilization, a hot air oven capable of being maintained at (180^{+5}_{0}) °C for a minimum holding time of 30 min, at (170^{+5}_{0}) °C for a minimum holding time of 1 h or at (160^{+5}_{0}) °C for a minimum holding time of 2 h.

2) Disposable equipment is an acceptable alternative to reusable glassware.